REMARKS

The specification has been amended to delete references to Figs. 10-14c, which figures were inadvertently omitted from the above-referenced patent application as originally filed. The drawings are not necessary for understanding the invention.

No new matter has been added by this amendment.

No fees are believed to be due. However, the Commissioner is authorized to charge any additional fees that may be due, or credit any overpayment of same, to Deposit Account No. 50-0311, Reference No. 21486-032.

Respectfully submitted,

Ingrid A. Beattie, Reg. No. 42,306
c/o MINTZ, LEVIN
One Financial Center
Boston, Massachusetts 02111
Tel: (617) 542-6000
Fax: (617) 542-2241

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APPENDIX: Marked up version of amendments

In the specification:

On page 16, before "Detailed Description", delete lines 1 – 15:

[Fig. 10 is a diagram showing AAH cDNA and the location at which antisense oligonucleotides bind. The locations shown are relative to the AUG start site of the AAH cDNA.

Fig. 11 is a photograph of an electrophoretic gel showing inhibition of AAH gene expression by antisense oligonucleotide DNA molecules.

Fig. 12 is a line graph showing AAH antisense oligonucleotide binding in neuroblastoma cells.

Fig. 13 is a bar graph showing inhibition of AAH gene expression as a result of AAH antisense oligonucleotide delivery into neuroblastoma cells.

Fig. 14A is a photograph of a Western blot assay expression of NOTCH proteins.

Fig. 14B is a photograph of an electrophoretic gel showing Hes-1 gene expression as measured by reverse transcriptase - polymerase chain reaction (RT-PCR).

Fig. 14C is a photograph of a Western blot assay showing expression of NOTCH-1 and Jagged-1 under conditions in which IRS-1 signalling is reduced.]

Detailed Description

HAAH is a protein belonging to the alpha-ketoglutarate dependent dioxygenase family of prolyl and lysyl hydroxylases which play a key role in collagen biosynthesis. This molecule hydroxylates aspartic acid or asparagine residues in EGF-like domains of several proteins in the presence of ferrous iron. These EGF-like domains contain conserved motifs, that form repetitive sequences

in proteins such as clotting factors, extracellular matrix proteins, LDL receptor, NOTCH homologues, or NOTCH ligand homologues.

The alpha-ketoglutarate-dependent dioxygenase aspartyl (asparaginyl) beta-hydroxylase (AAH) specifically hydroxylates one aspartic or asparagine residue in EGF-like domains of various proteins. The 4.3-kb cDNA encoding the human AspH (hAspH) hybridizes with 2.6 kb and 4.3 kb transcripts in transformed cells, and the

On page 28, lines 16 through 17, delete the sentence beginning "Fig. 14A. shows..." and ending with "Western blot.".

On page 28, lines 23 to 24, delete the sentence beginning at "Expression of the Hes-1..." and ending with "(Fig. 14B).".

On page 28, line 27, delete the sentence beginning with "Fig. 14C" and ending with "(hNOTCH-1) and".

developing cancer or an individual who has previously been diagnosed with cancer. For example, the antibodies are useful to diagnose metastases of a tumor, which has been surgically excised or treated by chemotherapeutic or radiotherapeutic methods. The sensitivity of the method is sufficient to detect micrometastases in tissues such as lymph nodes. Early and sensitive diagnosis of tumors in this manner allows prompt therapeutic intervention.

The labeled antibody is administered to an individual using known methods, e.g., intravenously, or direct injection into solid or soft tissues. The antibody is allowed to distribute throughout the tissue or throughout the body for a period of approximately 1 hour to 72 hours. The whole body of the individual is then imaged using methods known in the art. Alternatively, a small portion of the body, e.g., a tissue site suspected of harboring a tumor, is imaged. An increase in antibody binding, as measured by an increase in detection of the label, over the level of baseline binding (to normal tissue) indicates the presence of a tumor at the site of binding.

Activation of NOTCH signaling

NOTCH signalling is activated in cells highly expressing AAH. [Fig. 14A. shows the presence of a 110 kDa NOTCH fragment as revealed by using Western blot.] Overexpression of enzymatically active AAH is shown by a display of the 100 kDa cleaved, active NOTCH-1 (Lane 1, Mock DNA transfected clone; Lane 2, clones 7; and Lane 3, clone18). In contrast, NOTCH-2 was not activated. There was enhanced expression of the full length Jagged ligand in clones

expressing AAH as compared to the mock DNA transfected clone. Tubulin was used as internal control for protein loading.

[Expression of the Hes-1, a known downstream effector gene, is activated by NOTCH signaling (Fig. 14B).] Only AAH-expressing clones activate Notch expression as a transcription factor and subsequently unregulates Hes-1 gene expression as revealed by competitive RT-PCR. Lower panel is an RT-PCR product of GAPDII that served as internal control. [Fig. 14C shows expression of human NOTCH-1 (hNOTCH-1) and]

On page 29, line 1, delete the first sentence beginning with "Jagged-1..." and ending with mutant (DhIRS-1).":

[Jagged-1 where IRS-1 signaling is reduced by a dominant negative mutant (DhIRS-1).] Such cells demonstrate downregulation AAH expression and demonstrate a parallel decrease in NOTCH-1 and Jagged levels by Western blot analysis. Tubulin was used as an internal control for protein loading.

Methods of identifying compounds that inhibit HAAH enzymatic activity

Aspartyl (asparaginyl) beta-hydroxylaseydroxylase (AAH) activity is measured *in vitro* or *in vivo*. For example, HAAH catalyzes posttranslational modification of β carbon of aspartyl and asparaginyl residues of EGF-like polypeptide domains. An assay to identify compounds which inhibit hydroxylase activity is carried out by comparing the level of hydroxylation in an enzymatic reaction in which the candidate compound is present compared to a parallel reaction in the absence of the compound (or a predetermined control value). Standard hydroxylase assays carried out in a testtube are known in the art, e.g., Lavaissiere et al., 1996, J. Clin. Invest. 98:1313-1323; Jia et al., 1992, J. Biol. Chem. 267:14322-14327; Wang et al., 1991, J. Biol. Chem. 266:14004-14010; or Gronke et al., 1990, J. Biol. Chem. 265:8558-8565. Hydroxylase activity is also measured using carbon dioxide ($^{14}CO_2$ capture assay) in a 96-well microtiter plate format (Zhang et al., 1999, Anal. Biochem. 271:137-142. These assays are readily automated and suitable for high throughput screening of candidate compounds to identify those with hydroxylase inhibitory activity.

Candidate compound which inhibit HAAH activation of NOTCH are identified by detecting a reduction in activated NOTCH in a cell which expresses or overexpresses HAAH, e.g., FOCUS HCC cells. The cells are cultured in the presence of a candidate compound. Parallel cultures are incubated in the absence of the candidate compound. To evaluate whether the compound inhibits HAAH activation of NOTCH, translocation of activated NOTCH to the nucleus of the

cell is measured. Translocation is measured by detecting a 110 kDa activation fragment of NOTCH in the nucleus of the cell. The activation fragment is cleaved from the large (approximately 300 kDa)

On page 58, lines 13-15, delete the entire paragraph.

Table 5: Sequence of exemplary oligonucleotide molecules

Location (-1)
5' CAT TCT TAC GCT GGG CCA TT 3' (SEQ ID NO:10)

Location (-6)
5' TTA CGC TGG GCC ATT GCA CG 3' (SEQ ID NO:11)

Location (-11)
5' CTG GGC CAT TGC ACG GTC CG 3' (SEQ ID NO:12)

Sense
5' ATC ATG CAA TGG CCC AGC GTA A 3' (SEQ ID NO:13)

[Fig. 10 shows the region of the AAH gene to which the antisense oligonucleotides described in Table 5 bind. All of the oligonucleotides were designed using MacVector 6.5.3 software.]

AAH antisense oligonucleotides tested were found to inhibit AAH gene expression. Using an *in vitro* cell free transcription translation assay (TNT Quick Coupled System), the human AAH cDNA (pHAAH) was used to synthesize AAH protein. *In vitro* translation was achieved with rabbit reticulocyte lysate included in the reaction mixture. The translated product was labeled with [^{35}S] methionine in the presence of reaction buffer, RNA polymerase, amino acid mixture, and ribonuclease inhibitor (RNAsin). The products were analyzed by SDS-PAGE followed by autoradiography. A luciferase (Luc) expressing plasmid was used as a positive control. In the second and third lanes, synthesis of the ~85 kD AAH protein is shown (AAH, arrow) using 1 or 2 micrograms of plasmid as the template and the T7 DNA-dependent RNA polymerase primer/promoter to generate mRNA. The addition of 100x or 1000x excess antisense oligonucleotide primer resulted in progressively greater degrees of inhibition of AAH protein synthesis, whereas the inclusion of the same amounts of sense oligonucleotide had no effect on

AAH protein synthesis. Further studies demonstrated complete inhibition of AAH protein synthesis only with the antisense oligonucleotides.

On page 59, lines 2-5, delete the sentence beginning with "Fig. 11..." and ending with "HAAH protein.".

On page 59, lines 6-8, delete the sentence beginning with "Fig. 11..." and ending with "inside cells.".

In addition, effective inhibition of gene expression was observed using all three antisense oligonucleotides tested. [Fig. 11 shows the results of an *in vitro* transcription/translation analysis of AAH antisense oligonucleotides and shows that the antisense oligonucleotides tested block translation of the HAAH RNA and subsequent protein synthesis of HAAH protein.]

Inhibition of AAH gene expression was also tested in cells. [Fig. 11 shows the results of a Microititer In situ Luminescence Quantification (MILQ) Assay and demonstrates the actual effect of the antisense oligonucleotides inside cells.] Substantial reduction in HAAH gene expression was detected by simply adding the antisense oligonucleotides to the culture medium of the cells. The MILQ assay quantifies *in situ* hybridization binding in cultured cells without the need for RNA extraction. The MILQ assay was used to study competitive antisense binding inhibition to illustrate that the antisense probe hybridized to the mRNA expressed endogenously within the Sh-SySy neuroblastoma cells. In this figure, inhibition of FITC-labeled Location -6 antisense oligonucleotide binding using specific unlabeled antisense oligonucleotides is shown. Minimal inhibition of binding was observed using non-relevant oligonucleotides. The unlabeled specific oligonucleotide was capable of effectively competing for the binding site designated by the FITC-conjugated Location -6 probe, whereas the non-relevant probe exhibited significantly less inhibition at the same molar concentration. Bound probe (FITC-labeled) was detected using horseradish peroxidase conjugated antibodies to FITC, and luminescence reagents were used to detect the bound antibody. Luminescence units were corrected for cell density and are

arbitrary in nature. These data indicate that cells effectively take up antisense oligonucleotides in the surrounding environment and that the oligonucleotides taken up effectively and specifically inhibit HAAH gene expression.

Inhibition of HAAH gene expression is enhanced by contacting cells with a phosphorothioate derivative of the HAAH antisense. Phosphorothioate antisense

On page 60, lines 1-3, delete the sentence beginning with "Fig. 13..." and ending with "neuroblastoma cells."

derivatives are made using methods well known in the art. [Fig. 13 shows inhibition of AAH gene expression due to antisense (Location -6) oligonucleotide gene delivery into SH-SySy neuroblastoma cells.] The MILQ assay was used to measure gene expression resulting from antisense oligonucleotide gene delivery. Cells were contacted with AAH Location -6 antisense DNA, and AAH protein expression was measured using methods known in the art, e.g., the MICE assay (de la Monte, et al, 1999, Biotechniques), to determine if it was inhibited by hybridization with the oligonucleotide. The MICE assay is used to measure immunoreactivity in cultured cells without the need to extract proteins or perform gel electrophoresis. This assay is more sensitive than Western blot analysis. Using the MICE assay, AAH immunoreactivity was assessed in cells transfected with non-relevant (random) oligonucleotide sequences, specific antisense oligonucleotides (Location -6), and a phosphorothioate Location -6 antisense oligonucleotide. Phosphorothioate chemical modification of the oligonucleotide was found to permit greater stability of the DNA inside the cell since the sulfur group protects the DNA from the degradation that normally occurs with. phosphodiester bonds and cellular nucleases. Antisense AAH oligonucleotide (Location -6) transfection resulted in reduced levels of AAH immunoreactivity, and using the phosphorothioate linked Location -6 antisense oligonucleotide, the effect of inhibiting AAH gene expression was substantial relative to the levels observed in cells transfected with the random oligonucleotide. The more effective inhibition of AAH expression using the phosphorothioate-linked antisense oligonucleotide was likely due to the greater stability of the molecule combined, with retained effective binding to mRNA.

Example 7: Human IRS-1 mutants

Insulin/IGF-1 stimulated expression of HAAH in HCC cell lines. Dominant-negative IRS-1 cDNAs mutated in the plextrin and phosphotryosine

(PTB) domains, and Grb2, Syp and PI3K binding motifs located in the C-terminus of the molecule were constructed. Human IRS-1 mutant constructs were generated to evaluate